

FORM PTO-1390  TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE  ATTORNEY'S DOCKET NUMBER  4121-136
INTERNATIONAL APPLICATION NO.  PCT/EP00/07835 07835		U.S. APPLICATION NO. (If known, see 37 CFR 1.5)  <b>10/069056</b>
INTERNATIONAL FILING DATE  11 August 2000		PRIORITY DATE CLAIMED  13 August 1999
TITLE OF INVENTION  PARVOVIRUS NS 1 VARIANTS		
APPLICANT(S) FOR DO/EO/US  NUESCH, Jurg; ROMMELAERE, Jean		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li>   <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li>a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li>   <li>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</li>   <li>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li>   <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li>   <li>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).*(<b>Unsigned</b>)</li>   <li>10. <input checked="" type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol>		
<b>Items 11. to 16. below concern other document(s) or information included:</b> <ol style="list-style-type: none"> <li>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.               <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</li> <li>14. <input type="checkbox"/> A substitute specification.</li> <li>15. <input checked="" type="checkbox"/> A small entity statement.</li> <li>16. <input type="checkbox"/> Other items or information:</li> </ol>		

NOTE: This application is being filed with an unsigned Oath or Declaration under the provisions of 37 CFR § 1.53 in order that applicants may secure a filing date of February 11, 2002. Upon receipt of a "Notice to File Missing Parts - Filing Date Granted," an executed Declaration and Power of Attorney, will be filed in the Patent and Trademark Office. The undersigned agent affirmatively states that she has been duly authorized and appointed to file this application on behalf of the applicants and that the Declaration and Power of Attorney to be filed hereafter will confirm the undersigned agent's authorization and appointment. Applicants are entitled to small entity status within the meaning of 37 CFR § 1.9.

10/069056

17. <input checked="" type="checkbox"/> The following fees are submitted: <b>Basic National Fee</b> (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO .....\$860.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) .....\$0.00  No International preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .....\$0.00  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO .....\$1000.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .....\$0.00				CALCULATIONS	PTO USE ONLY																
				<b>JC13 Rec'd PCT/PTO 11 FEB 2002</b>																	
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				\$ 860.00																	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$																	
<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%;">Claims</td> <td style="width: 25%;">Number Filed</td> <td style="width: 25%;">Number Extra</td> <td style="width: 25%;">Rate</td> </tr> <tr> <td>Total Claims</td> <td>18-20 =</td> <td>0</td> <td>X \$18.00</td> </tr> <tr> <td>Independent Claims</td> <td>2- 3 =</td> <td>0</td> <td>X \$80.00</td> </tr> <tr> <td colspan="2">Multiple dependent claim(s) (if applicable)</td> <td></td> <td>+ \$270.00</td> </tr> </table>				Claims	Number Filed	Number Extra	Rate	Total Claims	18-20 =	0	X \$18.00	Independent Claims	2- 3 =	0	X \$80.00	Multiple dependent claim(s) (if applicable)			+ \$270.00	\$	
Claims	Number Filed	Number Extra	Rate																		
Total Claims	18-20 =	0	X \$18.00																		
Independent Claims	2- 3 =	0	X \$80.00																		
Multiple dependent claim(s) (if applicable)			+ \$270.00																		
<b>TOTAL OF ABOVE CALCULATIONS =</b>				860.00																	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$ 430.00																	
<b>SUBTOTAL =</b>				\$ 430.00																	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$																	
<b>TOTAL NATIONAL FEE =</b>				\$ 430.00																	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property				\$																	
<b>TOTAL FEE ENCLOSED =</b>				\$ 430.00																	
				<b>Amount to be:</b>	\$																
				<b>refunded</b>																	
				<b>Charged</b>	\$																
a. <input checked="" type="checkbox"/> A check in the amount of <u>\$430.00</u> to cover the above fees is enclosed.  b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.  c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>08-3284</u> . A duplicate copy of this sheet is enclosed.																					
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not yet been met, a petition to revive (37 CFR 1.127(a) or (b)) must be filed and granted to restore the application to pending status.</b>																					
SEND ALL CORRESPONDENCE TO:  <b>Steven J. Hultquist</b> <b>Intellectual Property/Technology Law</b> <b>P. O. Box 14329</b> <b>Research Triangle Park, NC 27709</b>																					
 <b>MARIANNE FUERER</b> <b>Registration No. 39,983</b>																					

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**In re Application of:** Jurg Nuesch, et al.  
**Application No.:** New U.S. National Stage Application of  
PCT International Application No. PCT/EP00/07835  
**International Filing Date:** 11 August 2000  
**Priority Date Claimed:** 13 August 1999 (European Appl. No. 99 115 161.4)  
**U.S. National Phase Filing Date:** Date of mailing identified below  
**Title:** PARVOVIRUS NS 1 VARIANTS



23448

PATENT TRADEMARK OFFICE

EXPRESS MAIL CERTIFICATE

I hereby certify that I am mailing the attached documents to the  
Commissioner for Patents on the date specified, in an envelope  
addressed to the Commissioner for Patents, Box Patent  
Application, Washington, DC 20231, and Express Mailed  
under the provisions of 37 CFR 1.10.

Katrina Holland

Name of Person Mailing This Document

Katrina Holland

Signature

February 11, 2002

Date

EV037733273US

Express Mail Label Number

PRELIMINARY AMENDMENT

Commissioner for Patents  
BOX PATENT APPLICATION  
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified new national phase patent application, please amend the application, as follows:

**In the Specification**<sup>1</sup>

Please insert on page 1, between the title of the application and the first paragraph, the following new paragraph:

**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application is filed under the provisions of 35 U. S.C. §371 and claims the priority of International Patent Application No. PCT/EP00/07835 filed August 11, 2000, which in turn claims priority of European Patent Application No. 99 115 161.4 filed August 13, 1999.

On page 4 please amend paragraphs 1, 2 and 3 to read as follows:

According to the invention parvovirus NS1 variants are preferred in which the shift of equilibrium is achieved by mutation of one or several phosphorylation sites. Particularly preferred are parvovirus NS1 variants which have a mutation at one or several of the phosphorylation sites 283, 363, 394 and 463. Even more preferred are the parvovirus NS1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8) and T463A (SEQ ID NO. 10), which are indicated in Table 1 and [figure 1] figures 1.1, 1.2, 1.3 and 1.4. In S283A, a serine is exchanged by an alanine at position 283, in T363A, a threonine is exchanged by alanine at position 363, in T394A a threonine is exchanged by alanine at position 394 and in T463A a threonine is exchanged by alanine at position 463.

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<sup>1</sup> Applicants has provided a marked-up version of amended paragraphs and claims 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 and 12 in Appendix A, and a clean set of all pending claims, amended to date, in Appendix B.

A further subject matter of the present invention relates to a nucleic acid, particularly a DNA, which codes for an above parvovirus NS1 variant. Such a DNA comprises preferably:

- (a) the DNA of fig. 1.1 (SEQ ID NO. 3), 1.2 (SEQ ID NO. 5), 1.3 (SEQ ID NO. 7) and 1.4 (SEQ ID NO. 9), respectively
- (b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or
- (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

The DNA of (a) was deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) on Aug. 11 1999, i.e. fig. 1.1 as Escherichia coli pRSV-NS: S283A under DSM 12994 (SEQ ID NO. 3), fig. 1.2 as Escherichia coli pRSV-NS: T363A under DSM 12995 (SEQ ID NO. 5), fig. 1.3 as Escherichia coli pRSV-NS: T394A under DSM 12996 (SEQ ID NO. 7) and fig. 1.4 as Escherichia coli pRSV-NS: T463A under DSM 12997 (SEQ ID NO. 9).

On page 7, please amend the last full paragraph to read as follows:

Fig. 1 shows the DNA and amino acid sequences of parvovirus NS1 variants according to the invention (fig. 1.1 (SEQ ID Nos. 3 and 4), 1.2 (SEQ ID Nos. 5 and 6), 1.3 (SEQ ID Nos. 7 and 8) and 1.4 (SEQ ID Nos. 9 and 10)) as compared to parvovirus NS1 wild type (SEQ ID Nos. 1 and 2). In this connection, the mutated sites in the parvovirus NS1 variants according to the invention are labeled each.

### **In the Claims**

Please amend claims 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 and 12 to read as follows:

1. A parvovirus NS 1 variant protein having a shifted equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b), wherein the shifted equilibrium is selected from the group consisting of:

- the activities (a) are reduced and eliminated, respectively, and activity (b) is maintained or increased; and
- activity (b) is reduced and eliminated, respectively, and the activities (a) are maintained or increased.

2. The parvovirus NS 1 variant protein according to claim 1, wherein one or several phosphorylation sites are mutated.
3. The parvovirus NS 1 variant protein according to claim 2, wherein the mutations are located at an amino acid residue site selected from the group consisting of: 283, 363, 394 and/or 463.
4. The parvovirus NS 1 variant protein according to claim 3, namely the NS 1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
5. A DNA, coding for the parvovirus NS1 variant protein according to claim 1.
6. The DNA according to claim 5, wherein the DNA comprises a member selected from the group consisting of :
  - (a) the DNA of SEQ ID Nos 3, 5, 7 and 9, said DNA comprising a mutated phosphorylation site,
  - (b) a DNA hybridizing with the DNA from (a) under high stringency conditions, said DNA comprising the mutated phosphorylation site of the DNA from (a), or
  - (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.
7. An expression vector, comprising the DNA according to claim 6.

9. A method of producing the parvovirus NS 1 variant protein according to claim 1, comprising the culturing of the transformant according to claim 8 under suitable conditions.
10. An antibody, directed against the parvovirus NS 1 variant protein according to claim 1.
11. A Kit comprising at least one member selected from the group consisting of:
  - (a) a parvovirus NS 1 variant protein according to claim 1,
  - (b) a DNA according to claim 5,
  - (c) an antibody according to claim 10, and
  - (d) conventional auxiliary agents, such as solvents, buffers, carriers markers and controls.
12. Use of the parvovirus NS 1 variant protein according to claim 1 as a toxin for treating tumoral diseases.

Please add claims 14-18.

14. The parvovirus NS 1 variant protein according to claim 3, wherein the NS 1 variants has a mutation at sites S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
15. A DNA, coding for the parvovirus NS1 variant protein according to claim 4.
16. An expression vector, comprising the DNA according to claim 5.
17. A method of producing the parvovirus NS 1 variant protein according to claim 4, comprising the culturing of the transformant according to claim 8 under suitable conditions.

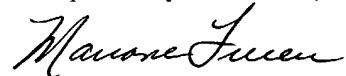
18. An antibody, directed against the parvovirus NS 1 variant protein according to claim 4.

**REMARKS**

A marked-up version of amended paragraphs and claims 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 and 12 is included herewith in Appendix A and a clean copy of all pending claims is included in Appendix B.

It is requested that the examination and prosecution of this application proceed on the basis of these amended claims 1-18.

Respectfully submitted,



Marianne Fuierer

Registration No. 39,983

Attorney for Applicants

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TECHNOLOGY LAW**  
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Attorney File: 4121-136

## APPENDIX A

### In the Specification

Please insert on page 1, between the title of the application and the first paragraph, the following new paragraph:

#### CROSS-REFERENCE TO RELATED APPLICATIONS

This application is filed under the provisions of 35 U. S.C. §371 and claims the priority of International Patent Application No. PCT/EP00/ 07835 filed August 11, 2000, which in turn claims priority of European Patent Application No. 99 115 161.4 filed August 13, 1999.

On page 4 please amend paragraphs 1, 2 and 3 to read as follows:

According to the invention parvovirus NS1 variants are preferred in which the shift of equilibrium is achieved by mutation of one or several phosphorylation sites. Particularly preferred are parvovirus NS1 variants which have a mutation at one or several of the phosphorylation sites 283, 363, 394 and 463. Even more preferred are the parvovirus NS1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8) and T463A (SEQ ID NO. 10), which are indicated in Table 1 and [figure 1] figures 1.1, 1.2, 1.3 and 1.4. In S283A, a serine is exchanged by an alanine at position 283, in T363A, a threonine is exchanged by alanine at position 363, in T394A a threonine is exchanged by alanine at position 394 and in T463A a threonine is exchanged by alanine at position 463.

A further subject matter of the present invention relates to a nucleic acid, particularly a DNA, which codes for an above parvovirus NS1 variant. Such a DNA comprises preferably:

- (a) the DNA of fig. 1.1 (SEQ ID NO. 3), 1.2 (SEQ ID NO. 5), 1.3 (SEQ ID NO. 7) and 1.4 (SEQ ID NO. 9), respectively
- (b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or
- (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

The DNA of (a) was deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) on Aug. 11 1999, i.e. fig. 1.1 as Escherichia coli pRSV-NS: S283A under DSM 12994 (SEQ ID NO. 3), fig. 1.2 as Escherichia coli pRSV-NS: T363A under DSM 12995 (SEQ ID NO. 5), fig. 1.3 as Escherichia coli pRSV-NS: T394A under DSM 12996 (SEQ ID NO. 7) and fig. 1.4 as Escherichia coli pRSV-NS: T463A under DSM 12997 (SEQ ID NO. 9).

On page 7, please amend the last full paragraph to read as follows:

Fig. 1 shows the DNA and amino acid sequences of parvovirus NS1 variants according to the invention (fig. 1.1 (SEQ ID Nos. 3 and 4), 1.2 (SEQ ID Nos. 5 and 6), 1.3 (SEQ ID Nos. 7 and 8) and 1.4 (SEQ ID Nos. 9 and 10)) as compared to parvovirus NS1 wild type (SEQ ID Nos. 1 and 2). In this connection, the mutated sites in the parvovirus Ns 1 variants according to the invention are labeled each.

### In the Claims

Please amend claims 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 and 12 to read as follows:

1. A parvovirus NS 1 variant protein having a shifted equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b), wherein the shifted equilibrium is selected from the group consisting of:
  - the activities (a) are reduced and eliminated, respectively, and activity (b) is maintained or increased; and [or]

- activity (b) is reduced and eliminated, respectively, and the activities (a) are maintained or increased.

2. The parvovirus NS 1 variant protein according to claim 1, wherein one or several phosphorylation sites are mutated.
3. The parvovirus NS 1 variant protein according to claim 2, wherein the mutations are located at an amino acid residue site [sites] selected from the group consisting of: 283, 363, 394 and/or 463.
4. The parvovirus NS 1 variant protein according to claim [2 or ]3, namely the NS 1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
5. A DNA, coding for the parvovirus NS1 variant protein according to claim 1. [any one of claims 1 to 4.]
6. The DNA according to claim 5, wherein the DNA comprises a member selected from the group consisting of:
  - (a) the DNA of [figure 1] SEQ ID Nos 3, 5, 7 and 9, said DNA comprising a mutated phosphorylation site,
  - (b) a DNA hybridizing with the DNA from (a) under high stringency conditions, said DNA comprising the mutated phosphorylation site of the DNA from (a), or
  - (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.
7. An expression vector, comprising the DNA according to claim [5 or] 6.
9. A method of producing the parvovirus NS 1 variant protein according to claim 1 [any one of claims 1 to 4], comprising the culturing of the transformant according to claim 8 under suitable conditions.

10. An antibody, directed against the parvovirus NS 1 variant protein according to claim 1 [any one of claims 1 to 4].
11. A Kit comprising at least one member selected from the group consisting of:
  - (a) a parvovirus NS 1 variant protein according to claim 1[the invention],
  - (b) a DNA according to claim 5 [the invention, e.g. an expression vector, particularly a parvovirus],
  - (c) an antibody according to claim 10, [the invention, as well as ] and
  - (d) conventional auxiliary agents, such as solvents, buffers, carriers markers and controls[,

[wherein of components (a) to (d) one or more representatives can be present each].
12. Use of the parvovirus NS 1 variant protein according to claim 1 [one of claims 1 to 4] as a toxin for treating tumoral diseases.

**APPENDIX B****In the Specification**

Please insert on page 1, between the title of the application and the first paragraph, the following new paragraph:

**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application is filed under the provisions of 35 U. S.C. §371 and claims the priority of International Patent Application No. PCT/EP00/07835 filed August 11, 2000, which in turn claims priority of European Patent Application No. 99 115 161.4 filed August 13, 1999.

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A further subject matter of the present invention relates to a nucleic acid, particularly a DNA, which codes for an above parvovirus NS1 variant. Such a DNA comprises preferably:

- (a) the DNA of fig. 1.1 (SEQ ID NO. 3), 1.2 (SEQ ID NO. 5), 1.3 (SEQ ID NO. 7) and 1.4 (SEQ ID NO. 9), respectively
- (b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or
- (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

The DNA of (a) was deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) on Aug. 11 1999, i.e. fig. 1.1 as Escherichia coli pRSV-NS: S283A under DSM 12994 (SEQ ID NO. 3), fig. 1.2 as Escherichia coli pRSV-NS: T363A under DSM 12995 (SEQ ID NO. 5), fig. 1.3 as Escherichia coli pRSV-NS: T394A under DSM 12996 (SEQ ID NO. 7) and fig. 1.4 as Escherichia coli pRSV-NS: T463A under DSM 12997 (SEQ ID NO. 9).

On page 7, please amend the last full paragraph to read as follows:

Fig. 1 shows the DNA and amino acid sequences of parvovirus NS1 variants according to the invention (fig. 1.1 (SEQ ID Nos. 3 and 4), 1.2 (SEQ ID Nos. 5 and 6), 1.3 (SEQ ID Nos. 7 and 8) and 1.4 (SEQ ID Nos. 9 and 10)) as compared to parvovirus NS1 wild type (SEQ ID Nos. 1 and 2). In this connection, the mutated sites in the parvovirus NS1 variants according to the invention are labeled each.

### **In the Claims**

1. A parvovirus NS 1 variant protein having a shifted equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b), wherein the shifted equilibrium is selected from the group consisting of:
  - the activities (a) are reduced and eliminated, respectively, and activity (b) is maintained or increased; and
  - activity (b) is reduced and eliminated, respectively, and the activities (a) are maintained or increased.

2. The parvovirus NS 1 variant protein according to claim 1, wherein one or several phosphorylation sites are mutated.
3. The parvovirus NS 1 variant protein according to claim 2, wherein the mutations are located at an amino acid residue site selected from the group consisting of: 283, 363, 394 and/or 463.
4. The parvovirus NS 1 variant protein according to claim 3, namely the NS 1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
5. A DNA, coding for the parvovirus NS1 variant protein according to claim 1.
6. The DNA according to claim 5, wherein the DNA comprises a member selected from the group consisting of :
  - (a) the DNA of SEQ ID Nos 3, 5, 7 and 9, said DNA comprising a mutated phosphorylation site,
  - (b) a DNA hybridizing with the DNA from (a) under high stringency conditions, said DNA comprising the mutated phosphorylation site of the DNA from (a), or
  - (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.
7. An expression vector, comprising the DNA according to claim 6.
8. A transformant, containing the expression vector according to claim 7.
9. A method of producing the parvovirus NS 1 variant protein according to claim 1, comprising the culturing of the transformant according to claim 8 under suitable conditions.

10. An antibody, directed against the parvovirus NS 1 variant protein according to claim 4.
11. A Kit comprising at least one member selected from the group consisting of:
  - (a) a parvovirus NS 1 variant protein according to claim 4,
  - (b) a DNA according to claim 5,
  - (c) an antibody according to claim 10, and
  - (d) conventional auxiliary agents, such as solvents, buffers, carriers markers and controls.
12. Use of the parvovirus NS 1 variant protein according to claim 1 as a toxin for treating tumoral diseases.
13. Use of the DNA according to claim 7 as a vector for gene therapy.
14. The parvovirus NS 1 variant protein according to claim 3, wherein the NS 1 variants has a mutation at sites S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
15. A DNA, coding for the parvovirus NS1 variant protein according to claim 4.
16. An expression vector, comprising the DNA according to claim 5.
17. A method of producing the parvovirus NS 1 variant protein according to claim 4, comprising the culturing of the transformant according to claim 8 under suitable conditions.
18. An antibody, directed against the parvovirus NS 1 variant protein according to claim 4.

6/Pr/5

JC13 Rec'd PCT/PTO 11 FEB 2002

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Parvovirus NS1 Variants

The present invention relates to parvovirus NS1 variants, DNAs coding for them and methods of producing the parvovirus NS1 variants. Furthermore, this invention concerns antibodies directed against the parvovirus NS1 variants as well as the use of the DNAs and the parvovirus NS1 variants.

Parvovirus designates a genus of the virus family Parvoviridae. The parvovirus genus comprises a number of small, icosahedric viruses that can replicate in the absence of a helper virus. Parvovirus contains a single-stranded DNA having a length of about 5.000 bp. At the 3' and 5' ends of the DNA there is one palindromic sequence each. The DNA codes for two capsid proteins, VP1 and VP2, as well as for two regulatory non-structure proteins, NS-1 and NS-2. The latter proteins are phosphorylated and show nuclear or both cytoplasmic and nuclear localization, respectively. NS1 is necessary for viral DNA replication and participates in the regulation of viral gene expression. Particularly, NS1 transactivates the promoter P38 and exhibits DNA-binding, helicase and DNA-nicking activities. Furthermore, NS1 induces cytotoxic and/or cytostatic stress in sensitive host cells.

Parvoviruses are usually well-tolerated by populations of their natural host, in which they persist without apparent pathological signs. This is due to both the protection of foetuses and neonates by maternal immunity, and the striking restriction of parvovirus replication to a narrow range of target proliferating tissues in adult animals. This host tolerance concerns especially rodent parvoviruses, for example the minute virus of mice (MVM) and H-1 virus in their respective natural hosts, namely mice and rats. In addition, humans can be infected with the latter viruses, without any evidence of

associated deleterious effects from existing epidemiological studies and clinical trials. On the other hand, it is known that certain parvoviruses, and especially rodent parvoviruses, are both oncotropic, i.e. accumulate preferentially in neoplastic versus normal tissues, and oncosuppressive, i.e. have a tumor-suppressive effect towards tumor cells, in various animal models. At least part of the oncosuppressive effect is thought to be due to a direct oncolytic action mediated by NS1. This oncosuppressive effect was also demonstrated against human tumor cells transplanted in recipient animals.

It is considered to use parvoviruses for therapeutic purposes. On the one hand, it seems to be of interest to use parvoviruses as vectors for therapeutic genes, i.e. for introducing such genes into the genome of cells. On the other hand, it is considered to use NS1 of parvoviruses as a toxin for treating tumoral diseases. However, initial experiments showed unsatisfactory results.

Therefore, it is the object of the present invention to provide a product by which parvoviruses and NS1 thereof, respectively, can be used for the above purposes.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's findings that it is possible to interfere with the activities of parvovirus NS1 so as to shift the equilibrium existing between the DNA replication and transcription activities (a) and the cytotoxicity activity (b). In particular, he produced parvovirus NS1 variants in which the DNA replication and transcription activities (a) are reduced and eliminated, respectively, whereas the cytotoxicity activity (b) is maintained or raised. Moreover, he produced parvovirus NS1 variants in which the cytotoxicity activity (b) is reduced and eliminated, respectively, whereas the DNA replication and transcription activities (a) are

maintained or raised. Examples of such parvovirus NS1 variants are indicated in Table 1 and figure 1. In addition, the applicant recognized that the above parvovirus NS1 variants and expression vectors coding for them, particularly parvoviruses, respectively, are suitable for therapeutic purposes.

According to the invention, the applicant's findings are used to provide a parvovirus NS1 variant in which the equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b) is shifted.

The expression "parvovirus" comprises any parvovirus, particularly a rodent parvovirus, such as minute virus of mice (MVM) and H-1 virus.

The expression "the equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b) is shifted" refers to the fact that in a parvovirus NS1 variant according to the invention such an equilibrium is shifted as compared to the parvovirus NS1 wild-type. In particular, the equilibrium can be shifted to the effect that the DNA replication and transcription activities (a) are reduced and eliminated, respectively, whereas the cytotoxicity activity (b) is maintained or raised. The cytotoxicity activity (b) can also be reduced and eliminated, respectively, whereas the DNA replication and transcription activities (a) are maintained or raised. Such an equilibrium can be determined by various methods. As regards the determination of the DNA replication activity, reference is made e.g. to methods described in Legendre and Rommelaere, 1992, J. Virol. 66, 5705; Cotmore et al., 1992, Virology 190, 365; Cotmore et al., 1993, J. Virol. 67, 1579, Cotmore and Tattersall, 1994, Embo. J. 13, 4145. As to the determination of the transcription activity reference is made to methods described e.g. in Rhode and Richards, 1987, J. Virol. 61, 2807. Regarding the determination of the cytotoxicity activity reference is made to the below examples.

According to the invention parvovirus NS1 variants are preferred in which the shift of equilibrium is achieved by mutation of one or several phosphorylation sites. Particularly preferred are parvovirus NS1 variants which have a mutation at one or several of the phosphorylation sites 283, 363, 394 and 463. Even more preferred are the parvovirus NS1 variants S283A, T363A, T394A and T463A, which are indicated in Table 1 and figure 1. In S283A, a serine is exchanged by an alanine at position 283, in T363A a threonine is exchanged by alanine at position 363, in T394A a threonine is exchanged by alanine at position 394 and in T 463A a threonine is exchanged by alanine at position 463.

A further subject matter of the present invention relates to a nucleic acid, particularly a DNA, which codes for an above parvovirus NS1 variant. Such a DNA comprises preferably:

- (a) the DNA of fig. 1.1, 1.2, 1.3 and 1.4, respectively
- (b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or
- (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

The DNA of (a) was deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) on Aug. 11, 1999, i.e. fig. 1.1 as *Escherichia coli* pRSV-NS:S283A under DSM 12994, fig. 1.2 as *Escherichia coli* pRSV-NS:T363A under DSM 12995, fig. 1.3. as *Escherichia coli* pRSV-NS:T394A under DSM 12996 and fig. 1.4 as *Escherichia coli* pRSV-NS:T463A under DSM 12997.

The expression "hybridizing DNA" refers to a DNA which hybridizes with a DNA from (a) under normal conditions, particularly at 20°C below the melting point of the DNA. In this connection, the expression "hybridizing" refers to conventional hybridization conditions, preferably to hybridization conditions where 5xSSPE, 1 % SDS, 1xDenhardt's solution are used as solution and the hybridization temperatures are between 35°C and

70°C, preferably 65°C. The hybridization is followed by a wash step first carried out with 2xSSC, 1 % SDS and then with 0.2xSSC at temperatures between 35°C and 70°C, preferably at 65°C. Furthermore, reference is made to Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, cold Spring Harbor NY (1989).

A DNA according to the invention can be present in a vector and expression vector, respectively. A person skilled in the art is familiar with examples thereof. In the case of an expression vector for *E. coli* these are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b, T7 based expression vectors and pQE-8. For the expression in yeast, e.g. pY100 and Ycpad1 have to be mentioned while e.g. pKCR, pEFBOS, cDM8, pMSCND, and pCEV4 have to be indicated for the expression in animal cells. The baculovirus expression vector pAcSGHisNT-A is especially suitable for the expression in insect cells.

In a preferred embodiment, the vector containing the DNA according to the invention is a virus, e.g. an adenovirus, vaccinia virus, an AAV virus or a parvovirus, such as MVM or H-1, a parvovirus being preferred. The vector may also be a retrovirus, such as MoMULV, MoMuLV, HaMuSV, MuMTV, RSV or GaLV.

For constructing expression vectors which contain the DNA according to the invention, it is possible to use general methods known in the art. These methods include e.g. *in vitro* recombination techniques, synthetic methods and *in vivo* recombination methods as described in Sambrook et al., *supra*, for example.

Furthermore, the present invention relates to host cells which contain the above described vectors. These host cells include bacteria, yeast, insect and animal cells, preferably mammalian cells. The *E. coli* strains HB101, DH1, x1776, JM101, JM109, BL21, XL1Blue and SG 13009, the yeast strain *Saccharomyces cerevisiae* and the animal cells L, A9, 3T3, FM3A, CHO, COS,

Vero, HeLa and the insect cells sf9 are preferred. Methods of transforming these host cells, of phenotypically selecting transformants and of expressing the DNA according to the invention by using the above described vectors are known in the art.

Moreover, the present invention relates to antibodies which specifically recognize an above described parvovirus NS1 variant, i.e. the region of the parvovirus NS1 variant where the mutation responsible for the shifted equilibrium, particularly a mutated phosphorylation site, is located. The antibodies can be monoclonal, polyclonal or synthetic antibodies or fragments thereof, e.g. Fab, Fv or scFv fragments. Preferably monoclonal antibodies are concerned. For the production it is favorable to immunize animals - particularly rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody - with an above parvovirus NS1 variant or with fragments thereof. Further boosters of the animals can be effected with the same parvovirus NS1 variant or with fragments thereof. The polyclonal antibody can then be obtained from the animal serum and egg yolk, respectively. The monoclonal antibody can be obtained according to standard methods, reference being made particularly to the method by Kähler and Milstein (Nature 256 (1975), 495) and Galfré (Meth. Enzymol. 73 (1981), 3). In this case, mouse myeloma cells are fused with spleen cells originating from the immunized animals. Antibodies according to the invention can be used in many ways, e.g. for the immunoprecipitation of the above described parvovirus NS1 variants or for the isolation thereof. The antibodies can be bound in immunoassays in liquid phase or to a solid carrier. In this connection, the antibodies can be labeled in various ways. The person skilled in the art is familiar with suitable markers and labeling methods. Examples of immunoassays are ELISA and RIA.

The present invention provides parvovirus NS1 variants in which the equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b) is shifted. In

particular, parvovirus NS1 variants are provided which have a reduced or no cytotoxicity activity, whereas the DNA replication and transcription activities are maintained or increased. Parvovirus NS1 variants are also provided in which the DNA replication and transcription activities are reduced and eliminated, respectively, whereas the cytotoxicity activity is maintained or raised. Thus, the present invention provides products which are suitable for therapeutic purposes. In particular, expression vectors according to the invention, e.g. parvoviruses, can be used for gene-therapeutic measures. Moreover, parvoviruses NS1 variants according to the invention are suitable as toxins, e.g. for treating tumoral diseases.

Therefore, a kit is also provided for the application of the present invention. This kit comprises the following:

- (a) a parvovirus NS1 variant according to the invention,
- (b) a DNA according to the invention, e.g. an expression vector, particularly a parvovirus,
- (c) an antibody according to the invention, as well as
- (d) conventional auxiliary agents, such as solvents, buffers, carriers, markers and controls.

Of component (a) to (d) one or more representatives can be present each.

#### Brief description of the drawings

Fig. 1 shows the DNA and amino acid sequences of parvovirus NS1 variants according to the invention (fig. 1.1, 1.2, 1.3 and 1.4) as compared to a parvovirus NS1 wild-type. In this connection, the mutated sites in the parvovirus NS1 variants according to the invention are labeled each.

The present invention is explained by the examples.

**Example 1: Preparation and purification of NS1 variants according to the invention**

The DNA of the NS1 variant S283A according to the invention was provided as an EcoRV to BstEII fragment obtained by chimeric PCR using two mutagenic primers. This fragment was then inserted into the corresponding cleaved expression vector pTHisNS1 (Nuesch et al., *Virology* 209, (1995), 122) to obtain pTHisNS1:S283A. Such a vector codes for a fusion protein comprising 6 histidine residues (N terminus partner) and S283A of Fig. 1 (C terminus partner). For expression and purification of S283A the NS1 gene under control of the bacteriophage T7 promoter was transferred into vaccinia virus and expressed in eucaryotic cells by double infection together with vTF7-3 (a vaccinia virus expressing the bacteriophage T7 DNA polymerase). 18 hrs post infection cells were harvested and nuclear extracts prepared. The histidine tagged S283A was then purified by affinity chromatography on Ni-NTA agarose and analyzed by 10 % SDS-PAGE (Nuesch et al., *supra*).

It showed that a parvovirus NS1 variant according to the invention can be prepared in highly pure form.

The NS1 variants T363A, T394A, and T463A were also produced and purified in the same way.

**Example 2: Preparation and detection of an antibody according to the invention**

Tubes were coated with purified NS1 variants prepared as in example 1 and monoclonal antibodies (e.g. scFv) specifically binding to S283A were isolated from human synthetic VH+VL scFV phage library (Griffith et al., *EMBO J.*, 13, (1994), 3245) according to standard panning protocols after >5 isolation and amplification procedures. The variable region of the isolated scFv harbored in the phagemid were sequenced to identify NS1

variant interacting partner proteins harboring such binding motifs from comparison with known genes in the gene bank.

It showed that monoclonal antibodies according to the invention can be isolated.

In addition, the NS1 variants were used for immunization of animals in order to obtain poly- or monoclonal antibodies.

**Example 3: Characterization of the parvovirus NS1 variants S283A, T363A, T394A and T463A according to the invention**

The characterization of the parvovirus NS1 variants comprised the determination of the DNA replication, transcription, cytotoxicity, DNA binding, nicking and helicase activities. Known methods were used for this purpose (cf. description, *supra*). As regards the determination of the helicase activity reference is made to Stahl et al. 1986, *EMBO J.* 5, 1999. As to the determination of the nicking activity reference is made to Christensen et al., 1997, *J. Virol.* 71, 1405 and Nuesch et al., 1995, *supra*. Regarding the determination of the DNA binding reference is made to Cotmore et al. 1995, *J. Virol.* 69, 1652. As far as the determination of the cytotoxicity activity is concerned, the following steps were carried out:

NS1 variants were transferred into an expression vector containing the NS1 gene under the control of the parvovirus MVMP4 promoter (genuine promoter driving the non-structural genes of MVM), and the green fluorescent protein (EGFP) under control of an additional promoter. These constructs were then transfected into A9 cells using lipofectamine (GibcoBRL) according to the manufacturer's instruction and the impact of the NS1 variant on the viability of the cells tested in time course experiments. Transfected cells were identified by fluorescence of the EGFP. Toxic effects were determined in comparison to wild type NS1 or a vector containing no NS1 gene

as a function of time as well as a measure of cytopathic changes on the cell morphology.

The data indicated in Table 1 were obtained:

Table 1

	S283A	T363A	T394A	T463A	wt
P38-TA	+	-	-	++++	++++
ACCA	+	++++	++	++	++
Nick-1	+	-	-	+++	+++
Nick-2	+++	-	-	++++	++++
Nick-3	++	-	-		++++
Heli	++	-	(+)	++++	++++
Rep	+	-	-	+	++++
Cyto	++++++	++	+++	(+)	+++

**Example 4: NS1 variants' expression after transduction using recombinant viral vectors**

NS1 expression cassettes containing the NS1 variants according to the invention under control of the parvoviral P4 promoter and a 3' untranslated region from parvovirus MVM to ensure stability and translation of the gene product, were transferred either in a parvovirus genome background as exemplified in example 3, or a heterologous viral genome background, such as vaccinia virus (example 1) or adenovirus. Promoter and terminator regions were exchanged according to the requirements. The nucleic acids containing the NS1 variants were then packaged either *in vivo* (after transient transfection into eucaryotic cells) or *in vitro* and the packaged transducing particles were isolated. These transducing units containing NS1 variants were used either for studies concerning gene regulation in tissue culture or animals, but also as therapeutic agents either alone or in combination with other agents (such as cytokines) in gene and cancer therapy

approaches.

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**Claims**

1. A parvovirus NS1 variant having a shifted equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b), wherein

5 - the activities (a) are reduced and eliminated, respectively, and activity (b) is maintained or increased or

10 - activity (b) is reduced and eliminated, respectively, and the activities (a) are maintained or increased.

2. The parvovirus NS1 variant according to claim 1, wherein one or several phosphorylation sites are mutated.

15 3. The parvovirus NS1 variant according to claim 2, wherein the mutations are located at sites 283, 363, 394 and/or 463.

20 4. The parvovirus NS1 variant according to claim 2 or 3, namely the NS1 variants S283A, T363A, T394A, and T463A.

25 5. A DNA, coding for the parvovirus NS1 variant according to any one of claims 1 to 4.

25 6. The DNA according to claim 5, wherein the DNA comprises:

(a) the DNA of figure 1,  
(b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or  
(c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

35 7. An expression vector, comprising the DNA according to

claim 5 or 6.

8. A transformant, containing the expression vector according to claim 7.

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9. A method of producing the parvovirus NS1 variant according to any one of claims 1 to 4, comprising the culturing of the transformant according to claim 8 under suitable conditions.

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10. An antibody, directed against the parvovirus NS1 variant according to any one of claims 1 to 4.

11. Kit comprising:

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(a) a parvovirus NS1 variant according to the invention,  
(b) a DNA according to the invention, e.g. an expression vector, particularly a parvovirus,  
(c) an antibody according to the invention, as well as  
(d) conventional auxiliary agents, such as solvents, buffers, carriers, markers and controls,

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wherein of components (a) to (d) one or more representatives can be present each.

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12. Use of the parvovirus NS1 variant according to any one of claims 1 to 4 as a toxin for treating tumoral diseases.

13. Use of the DNA according to claim 7 as a vector for gene therapy.

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(54) Title: PARVOVIRUS NS1 VARIANTS

(57) Abstract: The present invention relates to a parvovirus NS1 variant having a shifted equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b). Furthermore, this invention relates to DNAs coding for these parvovirus NS1 variants and methods of producing them. Additionally, this invention concerns antibodies directed against the parvovirus NS1 variants as well as the use of the DNAs and the parvovirus NS1 variants.

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Fig. 1

## Wild-type NS1

261 ATGGCTGGAAATGCTTACTCTGATGAAGTTGGGAGCAACCAACTGGTTAAAGGAAAAA 320  
 TACCGACCTTACGAATGAGACTACTTCAAAACCCCTCGTTGGTACCAATTCCCTTT  
 M A G N A Y S D E V L G A T N W L K E K -  
  
 321 AGTAACCAGGAAGTGTCTCATTTGTTTAAAGAAATGTTCAACTGAATGGAAAA 380  
 TCATTGGTCCTTCACAAGAGTAAACAAAATTTTACTTTACAAGTTGACTTACCTTT  
 S N Q E V F S F V F K N E N V Q L N G K -  
  
 381 GATATCGGAATGGAATAGTTACAAAAAGAGCTGCAGGAGGACGAGCTGAAATCTTACAA 440  
 CTATAGCCTACCTTATCAATGTTTCTCGACGTCCCTGCTCGACTTTAGAAATGTT  
 D I G W N S Y K K E L Q E D E L K S L Q -  
  
 441 CGAGGAGCGGAAACTACTGGGACCAAAGCGAGGACATGGAATGGAAACACAGTGGAT 500  
 CCTCCTCGCCTTGTGAAACCTGGTTCTCGCTCCCTGCTGACTTTAGAAATGTT  
 R G A E T T W D Q S E D M E W E T T V D -  
  
 501 GAAATGACCAAAAAGCAAGTATTCAATTGGATTCTTGGTTAAAGAAATGTTATTTGAA 560  
 CTTTACTGGTTTCTGTTCAAGTAAAACTAAGAAACCAATTTCACAATAAAACTT  
 E M T K K Q V F I F D S L V K K C L F E -  
  
 561 GTGCTAACACAAAGAATATATTCTGGTATGTTAATGGTTGTGCAACATGAATGG 620  
 CACGAATTGTGTTCTTATATAAGGACCACTACAATTAAACCAACACGTTGACTTAC  
 V L N T K N I F P G D V N W F V Q H E W -  
  
 621 GGAAAAGACCAAGGCTGCCACTGCCATGTTACTAATTGGAGGAAAGGACTTTAGTCAGCT 680  
 CCTTTCTGGTTCCGACCGTGACGGTACATGATTAAACCTCCCTGAAATCAGTTGCA  
 G K D Q G W H C H V L I G G K D F S Q A -  
  
 681 CAAGGGAAATGGTGGAGAAGGCAACTAAATGTTACTGGAGCAGATGGTTGGTAACAGCC 740  
 GTTCCCTTACCAACCTCTCCGTTGATTACAAATGACCTCGTACCAACCATTGTCGG  
 Q G K W W R R Q L N V Y W S R W L V T A -  
  
 741 TGTAATGTGCAACTAACCCAGCTGAAAGAATTAAACTAAGAGAAATAGCAGAAGACAAT 800

Fig. 1 (Fortsetzung I)

ACATTACACGTTGATTGTGCTCGACTTTCTTAATTGATTCTCTTATCGCTTCTGTTA  
 C N V Q L T P A E R I K L R E I A E D N -  
 GAGTGGGTTACTCTACTTACTTATAAGCATAAGCAAACCAAAAAAGACTATACCAAGTGT  
 801 CTCACCCATGAGATGAATATTGTTATTCGTTGGTTTTCTCATATGGTTACAA  
 E W V T L L T Y K H K Q T K K D Y T K C -  
 GTTCTTTGGAAACATGATTGCTTACTATTTTAACTAAAAAGAAAATAAGCACTAGT  
 861 CAAGAAAACCTTTGACTAACGAATGATAAAAATTGATTTCTTTATTCGTGATCA  
 V L F G N M I A Y Y F L T K K K I S T S -  
 CCACCAAGAGACGGAGGCTATTTCTTAGCAGTGACTCTGGCTGGAAAACTAACTTTA  
 921 GGTGGTTCTGCCTCCGATAAAAGAACATCGTCACTGAGACCGACCTTTGATTGAAAAT  
 P P R D G G Y F L S S D S G W K T N F L -  
 AAAGAAGGCGAGCGCCATCTAGTGAGCAAACATAACTGATGACATGCGGCCAGAACG  
 981 TTTCTTCCGCTCGCGGTAGATCACTGTTGATATGTGACTACTGTACGCCGGCTTTGC  
 K E G E R H L V S K L Y T D D M R P E T -  
 GTGAAACACAGTAACCACTGGCAGGAAACTAAGCGCGCAGAATTCAAACAA  
 1041 CAACCTTGGTGTCAATTGGTGACCGCTCTTGATTGGCGCTCTAAGTTGATTTTT  
 V E T T V T T A Q E T K R G R I Q T K K -  
 GAAGTTCTATTAAAACACTACCTAAACAGAGCTGGCATAAAAAGAGTAACCTCACCAAGAG  
 1101 CTTCAAAGATAATTGATGTCAATTCTCGACACGTATTCTCATGGAGTGCTC  
 E V S I K T T L K E L V H K R V T S P E -  
 GACTGGATGATGATGCAGCCAGACAGTTACATTGAAATGATGGCTCAACCAGGTGGAGAA  
 1161 CTGACCTACTACAGTCGGTCTGTCAACTGTAACCTTACTACCGAGTTGGTCCACCTCTT  
 D W M M M Q P D S Y I E M M A Q P G G E -  
 AACCTGCTAAAAATACGCTAGAGATTGACACTAACTCTAGCCAGAACCAAAACAGCA  
 1221 TTGGACGACTTTATGCGATCTAAACATGTGATTGAGATCGGTCTGGTTGCTCGT  
 N L L K N T L E I C T L T L A R T K T A -  
 TTTGACTTAATTAGAAAAAGCTGAAACAGCAAACTAACCAACTTCACTGCGCTGAC  
 1281 AAACGAAATTAAAATCTTTGACCTTGCTGTTGATGGTCAATTGAAAGTGTACGGACTG  
 F D L I L E K A E T S K L T N F S L P D -  
 ACAAGAACCTGCAGAATTGCTTTCATGGCTGAACTATGTTAAAGTTGCCATGCT  
 1341 TGTTCTGGACGTCTAAACGAAAAGTACCGACCTGATACAATTCAACGGTACCGA  
 T R T C R I F A E H G W N Y V K V C H A -

Fig. 1 (Fortsetzung II)

1401 ATTTGCTGTGTTAACAGACAGGAGGAAAAGAAACTGTTTATTCATGGACCA 1460  
 TAAACGACACAAAATTGCTGTCCTCCGTTCTTATGACAAAATAAGTACCTGGT  
 I C C V L N R Q G G K R N T V L F H G P -  
 GCCAGCACAGGCAAATCTATTGACAGCCATAGCACAACCAGTTGGCAATGTTGGT  
 1461 CGGTCTGTCCGTTAGATAATAACGTGTCGGTATCGTGTCTCAACCGTTACAACCA 1520  
 A S T G K S I I A Q A I A Q A V G N V G -  
 TGCTATAATGCAGCCAATGTAACCTTCCATTAAATGACTGTACCAACAAGAACTTGATT  
 1521 ACGATATTACGTCGGTACATTGAAAGGTAAATTACTGACATGGTGTCTGAACTAA 1580  
 C Y N A A N V N F P F N D C T N K N L I -  
 TGGGTAGAAGAAGCTGGTAACCTTGGACAGCAAGTAAACCAGTTAAAGCCATTGCTCT  
 1581 ACCCATCTTCTCGACCATTGAAACCTGTCGTTCAATTGGTCAAATTCTGGTAAACGAGA 1640  
 W V B E A G N F G Q Q V N Q F K A I C S -  
 GGTCAAACATTGCAATTGATCAAAAAGGAAAAGGCAGCAAACAGATTGAACCAACACCA  
 1641 CCAGTTGATAAGCGTAACTAGTTTCCCTTTCCGTCGTTGTCTAACTGGTTGTGGT 1700  
 G Q T I R I D Q K G K G S K Q I E P T P -  
 GTCATCATGACCACAAATGAGAACATTACAGTGGTCAGAATAGGCTGCGAAGAAACACCA  
 1701 CAGTAGTACTGGTGTACTCTGTAATGTCACAGTCTATCCGACGCTCTTCTGGT 1760  
 V I M T T N E N I T V V R I G C E E R P -  
 GAACACACTCAACCAATCAGAGACAGAACATGCTAACATTCACTAACACATACCTTGCT  
 1761 CTTGTTGAGTTGGTTAGTCTGTCTAACGAAATTGTAAGTAGATTGTATGGAACGGA 1820  
 E H T Q P I R D R M L N I H L T H T L P -  
 GGTGACTTTGGTTGGTTGACAAAATGAATGGCCCATGATTGCTGGTTGGTAAAG  
 1821 CCACTGAAACCAAAACCAACTGTTTACTTACCGGGTACTAAACACGAAACCAACATTG 1880  
 G D F G L V D K N E W P M I C A W L V K -  
 AATGGTTACCAATCTACCATGGCAAGCTACTGTCGTAATGGGCAAAGTCCGTGATTGG  
 1881 TTACCAATGGTTAGATGGTACCGTTGATGACACGATTACCCGTTCAAGGACTAAC 1940  
 N G Y Q S T M A S Y C A K W G K V P D W -  
 TCAGAAAATGGCGGAGCCAAACGTGCCACTCTTACGTTAAATTTACTAGGTTGGCACGC  
 1941 AGTCTTTGACCCGCCCTCGGTTCCACGGTTGAGGATATTAAATGATCCAAGCCGTGCG 2000  
 S E N W A E P K V P T P I N L L G S A R -  
 TCACCATTACGACACCGAAAAGTACGCCCTCTAGCCAGAACTATGCACTAACTCCACTT  
 2001 AGTGGTAAGTGCTGGCTTTCATGCCAGAGTCGGTCTGATACTGATTGAGGTGAA 2060

Fig. 1 (Fortsetzung III)

S P F T T P K S T P L S Q N Y A L T P L -  
2061 GCATCGGATCTCGAGGACCTGGCTTTAGAGCCTGGAGCACACCAAATACTCCTGTTGCG  
CGTAGCCTAGAGCTCTGGACCCAAATCTCGAACCTCGTGTGTTATGAGGACAAACGC 2120

A S D L E D L A L E P W S T P N T P V A -  
2121 GGCACTGCAGAAACCCAGAACACTGGGAGCTGGTCCAAAGCCTGCCAAGATGGTCAA  
CCGTGACGTCTTGGGTCTTGTGACCCCTCGACCAAGGTTCGGACGGTTCTACCACTT 2180

G T A E T Q N T G E A G S K A C Q D G Q -  
2181 CTGAGCCCAACTTGGTCAGAGATCGAGGAGGATTGAGAGCGTGTTCGGTGCAGAACCG  
GACTCGGGTTGAACCAAGTCTCTAGCTCCTCCTAAACTCTCGCACGAAGCCACGCCCTGGC 2240

L S P T W S E I E E D L R A C F G A E P -  
2241 TTGAAGAAAGACTTCAGCGAGCCGCTGAACTTGGACTAA 2279  
AACTTCTTCTGAAGTCGCTCGCGACTTGAACCTGATT

L K K D F S E P L N L D \* -

Fig. 1.1

1100 - 261 Wildtype-NS1-Sequence

1101 GAAAGTTCTATTAAAACCTACACTAAAGAGCTGGTGCATAAAAGAGTAACCTCACCAAGAG  
 CTTCAAGATAATTGATGTGAATTCTCGACCACGTATTTCTCATGGAGTGGTCTC 1160  
 E V S I K T T L K E L V H K R V T S P E -  
 → A S 283A

### 1161 - 2279 Wildtype-NS1-Sequence

Fig. 1.2

### 1340 - 261 Wildtype-NS1-Sequence

### 1401 - 2279 Wildtype-NS1-Sequence

Fig. 1.3

1400 - 261 Wildtype-NS1-Sequence

1401 ATTTGCTGTGTTAACAGACAAGGAGGCAAAAGAAATCTGTTTATTGATGGACCA  
 TAAACGACACAAAATTGCTGTCCTCCGTTCTTAAACAAAATAAAGTACCTGGT  
 I C C V L N R Q G G K R N T V L F H G P -  
 →A T394A

1461 - 2279 Wildtype-NS1-Sequence

Fig. 1.4

1640 - 261 Wildtype-NS1-Sequence

1641 GGTCAAACTATTGCAATTGATCAAAACGAAAAGGCAGCAGAACAGATTGAACCAACACCA  
 CCAGTTGATAAGCGTAACTAGTTTCCCTTCCGTCGTTGTCTAACTGGTTGTGGT  
 G Q T I R I D Q K G K G S K Q I E P T P -  
 →A T463A

1701 - 2279 Wildtype-NS1-Sequence

K 3039

## PATENT APPLICATION

DECLARATION AND POWER OF ATTORNEY  
FOR PATENT APPLICATION

ATTORNEY DOCKET NO. 4121-136

As a below named inventor, I hereby declare that:

My residence/post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PARVOVIRUS NS 1 VARIANTS

the specification of which is attached hereto unless the following box is checked:

(X) was filed February 11, 2002 as US Application Serial No. 10/069,056 or PCT International Application

Number \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose all information which is material to patentability as defined in 37 CFR 1.56.

## Foreign Application(s) and/or Claim of Foreign Priority

I hereby claim foreign priority benefits under Title 35, United States Code Section 119(a-d) or 365(b) of any foreign application(s) for patent or inventor(s) certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor(s) certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NUMBER	DATE FILED	PRIORITY CLAIMED UNDER 35 U.S.C. 119
EPO	99 115 161.4	13 August 1999	YES: <input checked="" type="checkbox"/> NO: _____
PCT	PCT/EP00/07835	11 August 2000	YES: <input checked="" type="checkbox"/> NO: _____

## U.S. Priority Claim

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NUMBER	FILING DATE	STATUS(patented/pending/abandoned)

## POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) listed below to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Steven J. Hultquist, Reg. No. 28021Marianne Fuierer, Reg. No. 39983

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(919) 419-9350

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00  
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Date

DECLARATION AND POWER OF ATTORNEY  
FOR PATENT APPLICATION (continued)

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